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REVIEW

NEURAMINIDASE INHIBITORS AS POTENTIAL ANTI-INFLUENZA DRUGS

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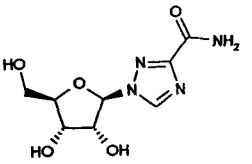
BACKGROUND

Influenza is a disease which most of us have encountered at first-hand, and the symptoms as a result of the respiratory tract infection are all too familiar; headache, catarrh, fever, aching limbs. The disease is generally extremely unpleasant and potentially fatal for the elderly and infirm. Complications including viral pneumonia and meningitis can occur, and as a result influenza is still a major killer in the developed world. Since the influenza virus undergoes frequent and rapid mutations in its surface antigens, its pathogenicity is characterised by epidemics and pandemics. In 1976 for example, 17000 excess deaths were recorded in England and Wales, and 26000 deaths were associated with the UK outbreak in 1989–90.[1] This is in addition to a general background mortality rate; for example in the USA, where the disease is still one of the 10 most common causes of death, 10000 people die each year as a result of influenza. As a result, although the subject of extensive interest,[2] vaccination is presently only moderately effective. Current vaccines are also unlikely to be effective against a new pandemic strain, whereas a good antiviral agent would have enormous potential in such situations. The significant human, economic and healthcare costs associated with the disease mean that intense efforts are being made to achieve this goal. The search for pharmaceutical agents has been greatly facilitated by major strides forward in knowledge of the virus and the virus-specific functions associated with its life-cycle. The virus particle consists of three major functional components[3] of potential interest for therapeutic intervention: (1) among the surface antigens

the haemagglutinin (HA) protein[4] is involved in recognition of host receptor and in membrane fusion prior to the subsequent penetration by the virus of the host cell to exploit the cellular machinery, (2) the virus contains 8 strands of RNA which require replication and translation, and (3) the role of the second major surface antigen, the neuraminidase (NA) enzyme (EC 3.2.1.18, sialidase, acylneuraminyl hydrolase) has been exploited and is the major subject of this review. Efforts in all three areas have been documented.[5]

Ribavirin (**1**), in the form of its triphosphate (RTP), as well as Foscarnet (**2**) have been shown to directly inhibit influenza RNA polymerase enzyme activity. RTP inhibits the GTP-dependent capping at the 5′-end of viral mRNA.[6] However, although a broad spectrum antiviral, including against both influenza A and B,[7] it has only a two to three fold therapeutic margin in the clinic[8] and so has not been licensed for influenza.

1,3,4-Thiadiazol-2-yl cyanamide LY217896 (**3**)[9] is currently undergoing clinical evaluation. Although its exact mode of action is unknown, a related compound, 2-amino-1,3,4-thiadiazole, appears to be glycosylated and phosphorylated to give at least a mononucleotide; IMP-dehydrogenase is ultimately inhibited, so reducing intracellular nucleotide pools. The observed lack of specificity of this compound[10] casts doubt on its clinical potential, however.



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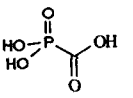

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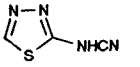

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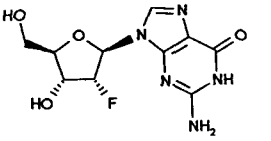
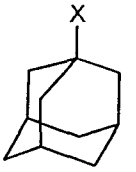


(4)

More recently, 2′-deoxy-2′-fluoroguanosine (2′-FdGuo) (**4**) has been shown[11] to be effective *in vitro* and in a mouse model against influenza A and B; the compound is phosphorylated by cellular kinases to its triphosphate, but its precise mechanism of action is yet to be reported. Detailed structural information for proteins involved in RNA processing is not available, however, making a fully rational approach to inhibitors more problematic.

The host-virus fusion process is addressed by amantadine (**5**) and its close analogue rimantidine (**6**), which remain the only compounds licensed for the prophylaxis and treatment of influenza A infection. These compounds are believed to block the ion channel function of the virus M2 protein[12] and so prevent the reduction in pH in the endocytosed cytoplasmic vacuole which is required for HA structural change and cleavage, resulting in virus-induced fusion. Neither (**5**) nor (**6**) is active against influenza B which lacks the M2 protein. Rapid emergence of reassortant strains of influenza has also been observed with the more extensively studied amantidine.[13]



(5) $X = NH_2$

(6) $X = CH(Me)NH_2$

An additional target in the cell-fusion process may be the agents involved in the HA cleavage itself.[14] Serine proteases are implicated in this process and inhibitors have been shown to be effective in suppressing influenza A *in vitro*[15] and *in vivo*.[16] However, this area is at an early stage of development.

The determination of the crystal structure[17,18] of the HA surface antigen offers the opportunity for a rational design approach to direct inhibition of the interaction of virus with its cell-surface, Neu5Ac-bearing ligand. The weak interaction of the protein with its natural ligand, and the likely multi-dentate virus-cell interaction[19] make this a potentially complicated task.

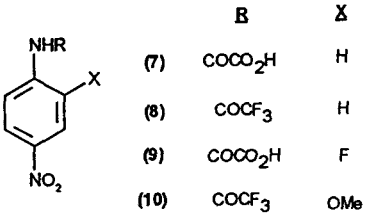
The influenza target which has generated the greatest level of excitement in recent years is the NA virion surface antigen.[20,21] NA enzymes are widespread among animals and a number of micro-organisms. They are glycohydrolases which cleave terminal α-ketosidically linked Neu5Ac from a wide array of glycobiomolecules.[22,23] Although somewhat more obscure than that of the other viral structural features, it is believed that the role of NA is in the assistance of release of newly formed virions from the surface of infected cells following budding[24,25] and of movement of virus through the mucus of the respiratory tract.[25,26]

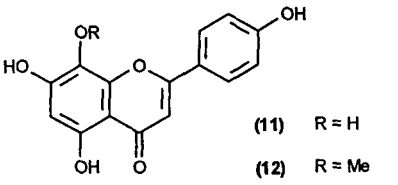
NEURAMINIDASE INHIBITORS

As a target, NA is highly attractive; its location on the surface of the virion and its point of action in the viral life cycle mean that inhibitor delivery problems are minimised; its enzymic nature make it intrinsically predisposed to inhibition at lower concentrations of small molecules, more so than the low affinity HA receptor; a great deal of work has been done in successfully inhibiting other glycohydrolases; and, perhaps above all, influenza NA has been extensively studied crystallographically and biochemically, making available a high level of structural information with which to design new drug candidates. As a result then, in addition to random screening, approaches to the discovery of NA inhibitors have included primarily those based on knowledge of the substrate and mechanism of the enzyme, and those based on a detailed knowledge of the enzyme active site. Related to this has been work based on anti-NA antibodies, which was reviewed recently by Colman.[21] The complex nature of antibody/antigen binding and the high antigenic variation observed for influenza NA, mean that small molecule inhibitors, interacting directly with the enzyme active site, are likely to remain the best hope for furnishing a drug for the treatment of influenza viruses.

SCREENING APPROACH

In spite of the great and rapid advances in knowledge of biological targets and in technology to help in the design of inhibitors of undesired processes, one of the most powerful means available to the medicinal chemist for identifying novel lead compounds is the high-throughput screen. The impact of combinatorial chemistry on discovery of drugs, including those targeted against the influenza NA, remains to be seen. Prior to the availability of crystallographic and biochemical information on the influenza NA enzyme, random screening was the primary mode of inhibitor discovery, but such an approach is still employed and continues to generate reports in the literature. Several low affinity and largely non-selective influenza NA inhibitors were identified shortly following the realisation of the importance of the enzyme; these included oxamic acid derivatives,[27] substituted β-aryl-α-mercaptoacrylic acids and benzimidazole derivatives.[28] Although non-selective, experience with the *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid (Neu5Ac2en) series of compounds (*vide infra*) suggests that selectivity can be introduced through prudent chemical manipulation. Vasella and coworkers[29] modified the originally reported[27] non-selective (**7**) to produce a series including (**8**)–(**10**). All were shown to be selective non-competitive inhibitors of *V. cholerae* NA interacting at a single site (K_i = 2.66–5.18 × 10^{-4} M). Alternative modifications to these early inhibitors may lead to selectivity for influenza NA, although no reports to this end have appeared.





Feedstocks for screening come not only from libraries of single chemical entities but also from microbial fermentation and from plant isolates and examples from these sources showing influenza NA inhibitory activity have also been reported. These include a crude isolate of unknown type from fermentation of *S. aureus*[30] and a polyphenolic complex isolated from *Geranium sanguineum*.[31] Surprisingly, the latter has an MIC of 5–12.5 $\mu g.mL^{-1}$, a therapeutic index of at least 20 and demonstrates *in vivo* protection in a mouse model of influenza. In addition, an extensive study of the NA and influenza inhibitory activity of a series of plant flavanoids has also been reported.[32–34] Structurally similar flavanones and chalcones proved inactive[32] indicating a specific structural requirement. Isocutellarein (**11**) and F36 (**12**) were the most potent *in vitro* (IC_{50} F36 = 55 μM *vs* A/PR/8/34; Neu5Ac2en IC_{50} = 220 μM),

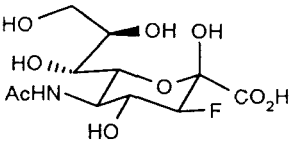
and *in vivo*[33] F36 prevented influenza proliferation in the mouse lung with "negligible" side-effects following intranasal (0.5 $mg.kg^{-1}$) or intraperitoneal (4 $mg.kg^{-1}$) administration. Isocutellarein was also active in the mouse model following oral administration. Both compounds are selective, but not specific, non-competitive inhibitors of influenza NA *in vitro*, although isocutellarein showed 0% inhibition of mouse liver NA at a concentration which inhibits influenza NA by 94%.

Optimism for such phenolic and polyphenolic compounds should be cautious, however, due to the generally known[35] non-selective binding of such compounds with proteins; indeed most screening protocols for plant isolates involve a selective removal of tannins prior to testing. The results of more extensive studies with such identified compounds will be of interest.

The level of reported success, then, in identifying influenza NA inhibitors through random screening approach is not high. Indeed, the greatest success has come from rational design of inhibitors based on mechanistic and/or structural information.

INHIBITOR DESIGN USING KNOWLEDGE OF MODE OF ENZYME ACTION

On a simple level, although as will be discussed later it greatly enhances the process, detailed knowledge of enzyme mechanism or structure is not required to commence design of NA enzyme inhibitors. Once it had been established[22,23] that influenza NA cleaves terminal Neu5Ac from glycosides to produce free Neu5Ac, a number of workers utilised this information in the design of potential inhibitors based on the substrate and on the product. In order to process such species at least some affinity of the enzyme for them must be required. Also, since such compounds are based on Neu5Ac, selectivity for NA's is likely to be greatly enhanced. The fluorinated Neu5Ac analogue 3FNA (**13**)[36] competitively inhibited influenza A virus NA with $K_{i\,app}$ = 8 μM, whilst DFNA (**14**) was inactive. 3FNA, which may be considered as a product analogue, was significantly more inhibitory (82% inhibition at 100 μM) than Neu5Ac (27% inhibition at 1 mM), but less so than Neu5Ac2en (92% at 100 μM). Although selective for NA's over other glycosidases, 3FNA also inhibits bacterial and mammalian NA's, but with a greater differential effect than Neu5Ac2en. 3FNA may constitute a starting point for further evaluation.



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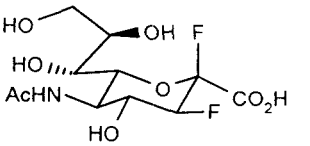
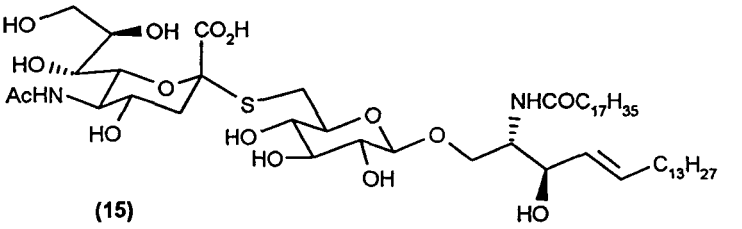


(14)

Whilst the enzyme may be predisposed to removal of NA product as rapidly as possible from the active site (K_i Neu5Ac is in mM range) its affinity for the substrate is likely to be greater. The challenge in synthesis of substrate analogues is to limit the molecular weight and synthetic complexity, and to introduce stability to the glycosidic linkage to prevent destruction by the enzyme of its potential inhibitor. Thus, as long ago as 1970, Khorlin *et al.*[37] reported effective but non-selective NA inhibition by small N- and S-glycosides of Neu5Ac. Fine-tuning, but also unfortunately with concomitant increase in size and complexity, of the thio-aglycone has been shown[38,39] to retain NA inhibitory activity and metabolic stability and improve selectivity for the influenza enzyme.



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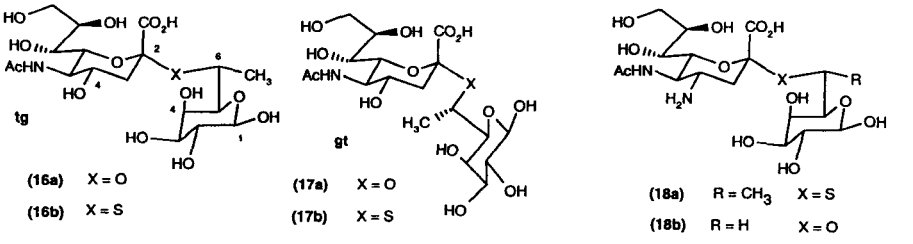
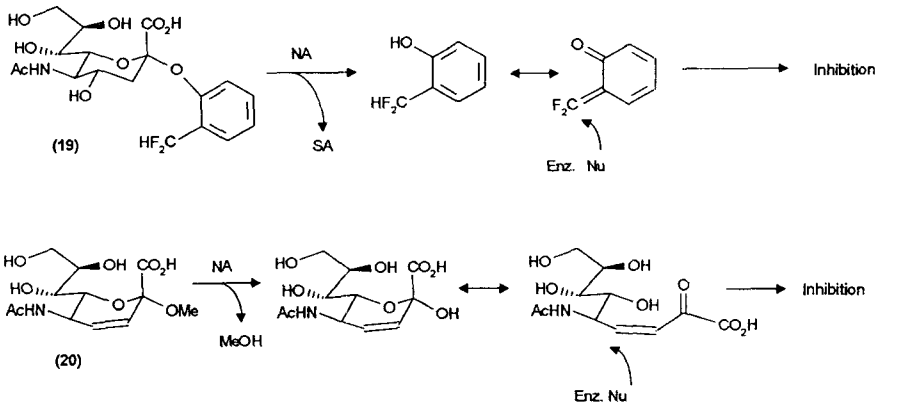
Thus, compound (**15**) competitively inhibits influenza A/PR/8/34 (H3/N2) enzyme with $K_i = 2.8 \times 10^{-6}$ M, 18 and 143 fold lower concentrations than for enzymes from *Cl. perfringens* and *Ar. ureafaciens*, respectively.[38] The inhibitory activity of this class of molecule was highly dependent on the nature of the aglycone; even inversion of the aglycone C4 stereochemistry of (**15**) reduced activity by 35 fold.[38] This dependence is vividly and elegantly borne out by Sabesan *et al.*[40] who showed that locking of the aglycone in either a *tg* or *gt* conformation by substitution at the C6 with a methyl group to give glycosides **16a**, **b** and **17a**, **b**, respectively, enabled recognition by influenza NA only of the *tg* derivatives. Thus, only O-glycoside **16a** and not **17a** was hydrolysed by influenza NA, and only **16b** and not **17b** was a competitive inhibitor (K_i = 0.3 mM). This was rationalised by molecular modelling of NMR-derived conformations into the reported crystal structure of the NA enzyme; only the *tg* conformers **16a**, **b** could be efficiently docked into the active site.[41]



The importance of the Neu5Ac 4-position in such analogues mirrors that of the 4-position of Neu5Ac2en transition state (TS) mimics (*vide infra*). Thus, incorporation[42] of an amine group (following the work of von Itzstein[43]) at C4 to give **18a** improves its inhibition of influenza NA (K_i = 50 μM). The incorporation of a guanidine group at this position is likely to be even more interesting.[43,44] It would also be interesting to examine the NA inhibitory activity of the simple 4-amino-Neu5Ac to determine whether the aglycone effect is of importance relative to that of the 4-substituent. Even the O-glycoside (**18b**) is a non-hydrolysable NA inhibitor (K_i = 150 μM), implying the 4-amino substituent prevents catalysis of the cleavage reaction, possibly through interaction with Asp151 of NA[43] (*vide infra*). Increasing the steric bulk at the galactose C1 position in analogues (**16a**) and (**16b**) to a $CH_2CH_2SiMe_3$ proved detrimental to inhibitory activity (K_i = 3.2 mM); however, an octadecyl glycoside is equipotent (K_i = 0.24 mM); the reason for this is unclear.[44]

The real advantage of such substrate inhibitors is that they have been proposed[44–46] as potentially capable of concomitantly functioning as HA inhibitors. As purely NA inhibitors the axial sialosyl carboxyl group is not optimally placed to interact with the three Arg residues in the NA active site[44] (*vide infra*). However, optimal interaction of specific functional groups of an inhibitor with the enzyme active site is not essential if the inhibitor is no longer dependent on equilibrium driven binding. Thus, if a selective targetting of the influenza NA can be achieved, a high level of inactivation by a low level of drug could be achieved by irreversible drug-NA binding. This is the approach that has been taken by the groups of Doutheau and Quosh who have reported[45,46] their initial exploratory work in this area. The designed mode of irreversible inhibition by compounds (**19**)[45] and (**20**)[46] are shown below. Compound (**19**) proved[45] to be a substrate for *C. perfringens* NA ($K_{i\,app}$ = 0.15 mM) and enzyme was inactivated irreversibly in a time and concentration dependent manner; its inhibitory activity against influenza NA is presently being examined. Compound (**20**), however, was a very weak (K_i = 7 mM) competitive inhibitor of influenza NA; it is, thus, poorly recognised by NA, and no irreversible binding is observed. Extensive work



in this field will be required if a drug is to be obtained. The toxicity risk associated with such an approach is also very high; even a low level release of the suicide substrate by just one of the many NA's present in the body would result in a species capable of reacting with any physiological nucleophile with potentially irreversible consequences.

TRANSITION STATE ANALOGUES

Enzymes are biological catalysts; as such they have evolved to decrease the energy of activation required in converting biological starting materials to products. To do so, they must greatly reduce the energy of the reaction transition state (TS). Enzyme active site amino acid residues will thus be so positioned as to be most effective in interacting with the TS or a compound closely similar to it. As a result compounds similar to substrate or product will never bind as efficiently to the enzyme as a TS mimic, and the latter class of compounds, therefore, offers the most appropriate starting point in design of NA inhibitors. This design process has been greatly enhanced by recent publications of crystal structures of various influenza NA subtypes, with and without bound substrates[21,43] as well as by detailed biochemical studies.[43,47] Thus, a great deal of structural and mechanistic detail on influenza NA is now available.

Based on substrate and product analogues a series of Neu5Ac2en analogues of Neu5Ac were first described by Meindl and Tuppy in 1969[48] including the parent Neu5Ac2en which was subsequently elaborated to a limited number of analogues including the 5-trifluoroacetamide analogue, FANA.[49] These compounds were non-selective inhibitors of NA's from viral, bacterial and mammalian sources[49] but were inhibitors of viral replication, causing aggregation of virus particles at the host cell surface.[50] However, they were not effective in animal models[51] following systemic administration, possibly in part due to poor pharmacokinetics and rapid renal clearance.[52] It was subsequently proposed[53] that, by analogy with other glycosidase enzyme mechanisms, the sialoside may be cleaved through a sialosyl cation TS, and that the geometry of this may be mimicked by the Neu5Ac2en analogues. Through X-ray and biochemical studies of various influenza virus enzyme subtypes, including detailed studies of the interactions of Neu5Ac and Neu5Ac2en with enzyme, the original general proposal was proved correct. The most recently proposed[43] mechanism of NA cleavage and the intricate network of interactions of the highly polar Neu5Ac with the overall polar influenza A NA active site[21] have been discussed in detail previously. These show that, in order for the Neu5Ac carboxyl to interact optimally in an equatorial arrangement with the active site Arg371, significant ring distortion from a $^2C_5$ chair solution conformation to a pseudo-boat conformation is required; the active site is of course set up to stabilise the sialosyl cation, and so the planar nature of Neu5Ac2en means its carboxyl can interact with Arg371 without conformational energy penalties.[41,54,55]

Despite poor overall sequence homology between some influenza NA subtypes (e.g. only 28% homology between A and B enzymes) the amino acid composition and 3D arrangement at the active site are remarkably similar. Whilst Colman[21] and others

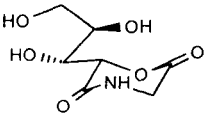
have pointed out the potential for mutation which could lead to resistance of influenza to inhibitors targeted at the NA active site, as has been observed for drugs aimed at other viral targets, the sequence homology between the various influenza NA's is an encouraging observation.

Neu5Ac2en (IC_{50} = 1.9 × 10^{-5}) and FANA (IC_{50} = 5 × 10^{-6}) have only modest non-selective activity, and so if this compound type were to be a drug candidate such issues needed to be addressed. Two general approaches have been followed; one based on empirical modifications to a Neu5Ac template; a second based on specific modifications to Neu5Ac2en dictated by computational and crystallographic results.

EMPIRICAL APPROACH

It has been reported[33] that 3-aza-4-oxo-*D-arabino*-octanoic acid 1,5-lactone (**21**) has activity against certain viral NA's comparable with that of Neu5Ac2en. However, the phosphonate, diphosphonate and sulphonate analogues of the open chain form (**22**) are virtually inactive against a variety of NA's.[56] Indeed, the reported[37] synthesis of (**21**) could not be repeated[57] and an unambiguous route to (**21**) showed it to rapidly decompose to the open chain form (**22**) which also proved inactive.[57]

Compound (**23**), however, demonstrated[58] comparable activity (85% inhibition at 100 μM) to Neu5Ac2en (95% inhibition at 100 μM) against influenza A NA, as well as against influenza A_2 and B enzymes. The furanose ring may ensure a more pseudo equatorial position of the carboxyl for TS mimicking than in Neu5Ac, without unsaturation as in Neu5Ac2en. The NHAc is provided from the sidechain rather than the sugar ring. The C4 epimer is less active as would be predicted. Although currently difficult to synthesise[58] (**23**) may afford an alternative starting point in drug design.



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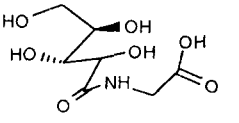

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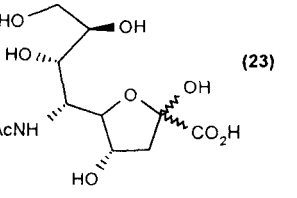

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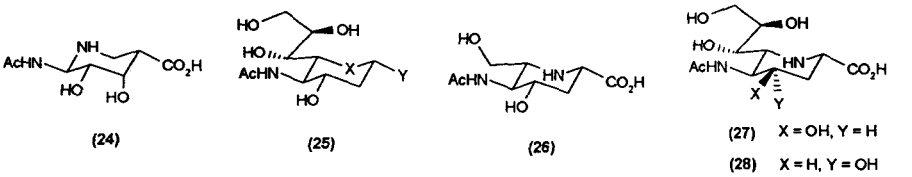
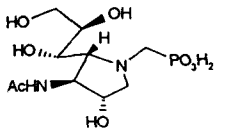
Neuraminidase inhibition has also been investigated as an objective in the search for antibacterial agents. Two classes of compound related to Neu5Ac templates which have been pursued primarily with a view to antibacterial activity are analogues of siastatin B (**24**),[59–63] and analogues (**25**) of Neu5Ac modified at the ring oxygen or the C2 centre.[56] It is important that information generated in pursuit of one therapeutic goal is not lost for application in pursuit of another. Indeed, compound (**25**), X = O, Y = $PO(OH)_2$, has been shown to also be a potent inhibitor of influenza NA (*vide infra*).

Also the active site architectures of viral and bacterial NA's have recently been shown to be remarkably similar.[64] Thus, it is important that all such compounds are tested against bacterial, viral and mammalian enzymes, whenever possible, if only to generate information on selectivity.

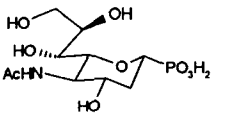


Siastatin B was isolated from *Streptomyces* culture. It inhibits NA isolated from *C. perfringens* and Streptococcus sp. (IC_{50} = 50 and 6.29 $\mu g.mL^{-1}$) at a similar level to Neu5Ac2en (IC_{50} = 12 and 1.97 $\mu g.mL^{-1}$) as well as inhibiting β-glucuronidase and *N*-acetyl-β-D-glucosaminidase. However, it has no activity against NA from *A. ureafaciens* or influenza virus A/Aichi/2/68 (H3N2). Neu5Ac2en has no activity against glucosidases or glucuronidases. Replacement of the acid functionality of (**24**) with CH_2NO_2 or CH_2NH_2, with or without OH at C2, resulted, not surprisingly, in loss of all NA inhibitory activity, but increased the glucosidase inhibitory activity.[61,62] On the other hand, changes to (**24**) to mimic more closely the structure of Neu5Ac2en increase activity against bacterial NA enzymes to a level which is greater than the activity observed for Neu5Ac2en. Thus, introduction of a substituent to mimic the C6 sidechain of Neu5Ac2en, or removal of the OH at the pseudo 3-position both increase activity, whilst inversion of stereochemistry at the pseudo C8 or C2 both eradicate activity against bacterial NA's. Interestingly, introduction of planarity around the acid has a detrimental effect; conformational effects on the N-containing ring may be unfavourable. No inhibitory data against influenza NA is reported for such compounds. Although siastatin B itself has an unfavourable spectrum of activity against influenza NA, such or related modifications may change its reported bacterial NA profile, and enable its use as a starting point for inhibitors of influenza NA.

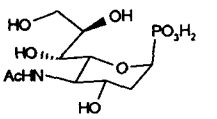
In a series similar in structure to siastatin B, examined as inhibitors of *Vibrio cholerae* NA, in which the ring nitrogen is one atom closer to the carboxyl bearing carbon, it was found[65] that compounds such as (**26**) ($K_i = 6.1 \times 10^{-3}$ M) were rather poor inhibitors. However, compounds with the full Neu5Ac2en C6 sidechain (**27**) and (**28**) possess 100 fold greater activity[65] similar to Neu5Ac2en itself. The analogous compound with a ring oxygen (X = H, Y = OH), akin to Neu5Ac is 100 fold less active.[65] In influenza NA binding the ring O of Neu5Ac appears to play no significant role in direct interaction;[43] thus, the beneficial effect of the nitrogen may be conformational, either of sidechains or the ring. Further substitution at position 2 of such compounds is detrimental to *V. cholerae* NA inhibitory activity.



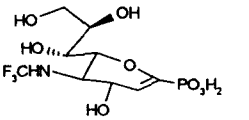
(29)



(30)



(31)



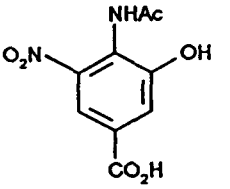
(32)

Compound (**29**) constitutes an additional template demonstrating good inhibition ($K_i = 5.3 \times 10^{-5}$ M) of *V. cholerae* NA.[66] Carboxylic acid-containing analogues of (**29**) were much less inhibitory. According to NMR, compound (**29**) has a different, perhaps unfavourable, conformation of its trihydroxypropyl sidechain relative to that of the trihydroxypropyl sidechain of Neu5Ac, suggesting[66] a remarkable effect of the phosphonate group on enzyme binding. Thus, compound (**30**) (*e*-PANA, $K_i = 5.5 \times 10^{-5}$ M)[67] was equipotent to (**29**) against *V. cholerae* NA and comparable to Neu5Ac2en ($K_i = 1.6 \times 10^{-5}$ M).[66] Interestingly, the axial phosphonate analog (**31**) (*a*-PANA) retains a surprisingly high level of activity against *V. cholerae* NA ($K_i = 2.3 \times 10^{-4}$ M) and may in part be due[66] to a favourable anomeric effect of P enabling a pseudo equatorial arrangement of the acid to be achieved relatively easily. The expected increase in activity on introduction of a C2-C3 double bond was not observed in this series; compound (**32**), an analogue of FANA, was a slightly worse inhibitor of *V. cholerae* NA ($K_i = 7.2 \times 10^{-5}$ M).[68] A recent report on these compounds demonstrates the validity of extrapolation of results from inhibition of NA's from other species to speculation for influenza NA; thus, compound (**30**) was shown[69] to be 100 fold more inhibitory of an N2 subtype of influenza A NA and influenza B/Lee/40 NA than Neu5Ac. Additionally, the X-ray crystal structures of *e*-PANA and *a*-PANA complexed to different influenza NA's were solved.[69] These demonstrated that the bound conformation of *e*-PANA was the same as its solution conformation, and so the energy penalty paid by Neu5Ac in binding to NA by adopting an appropriate conformation is not present in this compound. *a*-PANA on the other hand, undergoes a conformational change to place the $PO(OH)_2$ group in a pseudoequatorial position, and its activity is, thus, only comparable to that of Neu5Ac. That dehydro-analogue (**32**) is not more active may be due to the larger size[69] of $PO(OH)_2$ not being optimally accommodated in the active site as it is pushed closer to the protein by ring flattening.

Neu5Ac2en ANALOGUES – RATIONAL INHIBITOR DESIGN

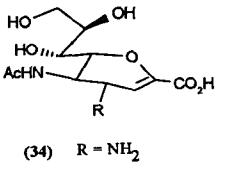
Although in some cases of similar activity to Neu5Ac2en, a major disadvantage of many of the compounds described above as starting templates for drug design is their synthetic complexity. On the other hand, Neu5Ac2en is accessible[70] from

Neu5Ac, in turn available in large quantities by enzymatic methods.[71] Some attempts have been made using the Neu5Ac2en/NA crystal structure to simplify the Neu5Ac2en template still further; *p*-acetamido-benzoic acid (**33**) (activity at 0.75 mM against influenza NA)[72] was designed, synthesised and then crystallised in the active site of NA and showed a similar binding mode to Neu5Ac2en. An iterative approach to the design of improved inhibitors of this type is now being taken.



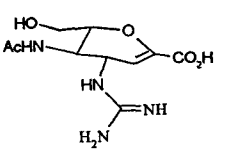
(33)

In the absence of alternative templates at the present time, however, Neu5Ac2en represents a starting point of moderate activity and synthetic accessibility from which to improve enzyme binding and selectivity. The most successful approach to this has again been through rational design using X-ray crystal structures and computational techniques.[21,43,73] Thus, in addition to the intricate H-bonding network, X-ray data in combination with the use of GRID software[74] indicates the potential benefit to be gained from the substitution of the 4-OH of Neu5Ac2en by an amino group through salt bridge formation with Glu119. The X-ray structure shows significant space in this region of the active site, and the larger, more basic guanidine group was also targeted, in anticipation of additional binding of this group to Glu227. Synthesis and testing of 4-amino-Neu5Ac2en (**34**) and 4-guanidino-Neu5Ac2en (**35**) confirmed the predictions of modelling. Thus, both (**34**) (K_i = 0.04 μM) and (**35**) (K_i = 0.001–0.0001 μM) were significantly more active[73] than Neu5Ac2en (K_i = 4 μM) against influenza A NA, as well as against influenza B NA. X-ray crystal structures for both compounds in the active site of influenza NA (reviewed in reference 43) demonstrate similar modes of binding as for Neu5Ac2en, and the confirmed interaction of the 4-amino-group with Glu119, as well as Asp151 and a hydrogen bond to a crystallographic water molecule. The H-bonding network of the guanidine group is even more extensive, binding also to Glu227 and the backbone carbonyl of Trp178; a significant binding energy is also probably achieved by expulsion of the water molecule observed in the crystal structure of the enzyme and (**34**). The expulsion of this water molecule is consistent with the

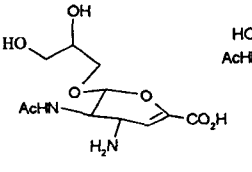


(34) R = NH_2

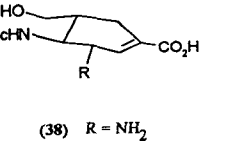
(35) R = $NHC(=NH)NH_2$



(36)



(37)



(38) R = NH_2

(39) R = $NHC(=NH)NH_2$

fact that (**35**) is a slow, tight-binding inhibitor[73] of influenza A NA (K_i = 0.03 nM),[75] an effect not observed with influenza B NA due possibly to a tighter binding of the equivalent water molecule in the active site.

Interestingly, both (**34**) and (**35**) are very much less active than Neu5Ac2en against NA's from bacterial and mammalian sources. Thus, whilst significant similarities exist between NA's from different sources[64,69] there are apparently sufficient differences, such as at the region around 4-OH of Neu5Ac2en in its bound state, to allow excellent selectivity in antiviral action.[77] The possible existence of other such selectivity "hotspots" in the influenza enzyme remains to be investigated. Whilst the possibility of mutation of influenza viral enzymes towards the structure of bacterial enzymes exists,[21,73] no wild strain of the virus has yet been found to have an NA resistant to these inhibitors.

The structure activity relationships (SAR) in this series of compounds is now being extensively investigated. Replacement of the substituent at C4 with hydrogen predictably increase the K_i drastically (8×10^{-2} mM).[76] Also, an extensive series of compounds with alternative nitrogen substituents at C4, similar to the 4-amino-[77] or the 4-guanidino-functions,[70] confirm the modelling predictions that the guanidine group is optimal for interaction at this region of the viral enzyme on a Neu5Ac2en template. In analogues modified at the C5 position, the 4-amino- and 4-guanidino-derivatives lacking a substituent at C5 demonstrate[78] significantly reduced anti-NA activity. Working with Neu5Ac2en as an inhibitor of viral NA, Meindl *et al.*[49] showed the critical importance of a small lipophilic group at this position; the subsequent crystal structural elucidations[43] indicate the close proximity of the Trp178 and the Ile222 sidechains, as well as H-bond acceptance through the acetyl carbonyl from Arg152 sidechain. The indications are[79] that the bacterial enzyme is similar in this region.

The X-ray structural studies also demonstrate interactions made by each of the three hydroxyls in the trihydroxypropyl C6-sidechain. Indeed, analogues of 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en with C6-H, C6-hydroxymethyl, and C6-dihydroxyethyl functionality[80] indicate a stepwise reduction in activity as each OH is removed. However, the hydroxymethyl derivative (**36**) with a 4-guanidino-group retains significant activity comparable to Neu5Ac2en; the 4-amino-analogue[80] is somewhat less active, whilst the analogues of this type with an OH or NHAc at the 4-position[76] are devoid of activity against influenza NA. An alternative attempt[81] at chemically simplifying the Neu5Ac type structure by use of a simple sugar starting material and an ether-linked C6-sidechain replacement furnished (**37**). However, this compound was devoid of NA or influenza inhibitory activity. The carbocyclic analogue of Neu5Ac has been shown[82] to be a weak NA inhibitor. The effect of replacing the ring oxygen of 4-amino- and 4-guanidino-derivatives with CH_2 has been investigated[83] through the synthesis of (**38**) and (**39**), allowing comparison with (**36**). Largely comparable levels of activity against NA enzyme were retained, confirming the structural studies[43] in which no significant interactions of the ring oxygen of 4-guanidino-Neu5Ac2en were observed. However, in *in vitro* plaque reduction assays, significant, beneficial effect for 4-amino-compound (**38**) was observed and may point to important effects of structural modifications on gross properties of such

NA inhibitors. As a result the carbocyclic analogues of 4-amino- and 4-guanidino-Neu5Ac2en are important synthetic targets.

In vitro anti-influenza activity of 4-guanidino-Neu5Ac2en ((**35**), GG167) has been shown[73,84,85] to correlate well with the NA inhibitory activity, with GG167 significantly more potent than Neu5Ac2en, amantidine and ribavirin. In human cells, anti-influenza A and B activity of GG167 was significantly greater than that of ribavirin;[85] also GG167 showed no inhibition of outgrowth of the human cells, whilst ribavirin did.[85] Whilst showing no activity against experimental infections of influenza A and B in ferrets[73] or in mice[86] when administered by the oral or intraperitoneal routes, it has been demonstrated that intranasal administration in either model has excellent effect. Thus, for example at a dose of 0.05 mg/kg twice daily in ferrets infected with influenza A, symptoms of fever were completely suppressed;[73] also in mice infected with influenza A 0.01 mg/kg twice daily reduced mortality and lung virus titres with no virus growing back following cessation of treatment.[86]

The potency of GG167 by the intranasal route, but lack of activity following administration by systemic routes in comparison with amantadine and ribavirin can be explained by bioavailability and pharmacokinetic properties, and by the fact that viral NA functions extracellularly where GG167 can exert its excellent *in vitro* activity.[86] Importantly, under conditions which normally rapidly give rise to amantadine resistance in patients, virus resistant to GG167 has not been isolated under pressure from the drug from animals in the laboratory. Clinical studies on GG167 with human volunteers and studies of the effect of GG167 on natural infections in humans, are ongoing.

In conclusion, a number of starting point templates based on knowledge of the influenza NA mechanism and structure have been identified as potential inhibitors of the enzyme. Of these the most potent is 4-guanidino-Neu5Ac2en, ((**35**), GG167). The enzyme inhibitory activity of GG167 has translated well into anti-influenza activity *in vitro*, and in *in vivo* animal models. It is hoped and anticipated that the ongoing clinical trials on this compound will finally establish the viability of neuraminidase inhibitors as anti-influenza drugs.

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